PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P5096PC01	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. PCT/IS2004/000011	International filing date *(day/month/year)* 27.08.2004	Priority date *(day/month/year)* 27.08.2003
International Patent Classification (IPC) or national classification and IPC C12N15/82, C12N15/62, C12N9/64		
Applicant ORF LIFTAEKNI EHF.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 10 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 1 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05.07.2005	31.10.2005
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized Officer Gurdjian, D Telephone No. +31 70 340-3388

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/IS2004/000011

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-27	as originally filed

Sequence listings part of the description, Pages

1, 2	as originally filed

Claims, Numbers

2-21	as originally filed
1	filed with telefax on 05.08.2005

Drawings, Sheets

1/3-3/3	as originally filed

☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-21
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-21
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-21
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this report has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed

   ☐ filed together with the international application in computer readable form

   ☒ furnished subsequently to this Authority for the purposes of search and/or examination

   ☒ received by this Authority as an amendment on10.12.2004

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional observations, if necessary:

Reference is made to the following documents :

D1: US-A-6 048 715 (HAYNES ET AL) 11 April 2000 (2000-04-11)

D2: WO 00/77174 A (CBD TECHNOLOGIES LTD; YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBRE) 21 December 2000 (2000-12-21)

D3: US 2002/164718 A1 (TCHAGA GRIGORIY S ET AL) 7 November 2002 (2002-11-07)

D4: WO 02/05922 A (MPB COLOGNE GMBH, MOLECULAR PLANT & PROTEIN BIOTECHNOLOGY; PETSCH, DAG) 24 January 2002 (2002-01-24)

D5: BORASTON ALISDAIR B ET AL: "A family 2a carbohydrate-binding module suitable as an affinity tag for proteins produced in Pichia pastoris" PROTEIN EXPRESSION AND PURIFICATION, vol. 21, no. 3, April 2001 (2001-04), pages 417-423, XP002321711 ISSN: 1046-5928

D6: BORASTON ALISDAIR B ET AL: "Binding specificity and thermodynamics of a family 9 carbohydrate-binding module from Thermotoga maritima xylanase 10A" BIOCHEMISTRY, vol. 40, no. 21, 29 May 2001 (2001-05-29), pages 6240-6247, XP002321709 ISSN: 0006-2960

D7: REEVES R A ET AL: "Sequencing and expression of additional xylanase genes from the hyperthermophile Thermotoga maritima FjSS3B.1." APPLIED AND ENVIRONMENTAL MICROBIOLOGY. APR 2000, vol. 66, no. 4, April 2000 (2000-04), pages 1532-1537, XP002321710 ISSN: 0099-2240

D8: CHHABRA S R ET AL: "Biochemical characterization of Thermotoga maritima endoglucanase Cel74 with and without a carbohydrate binding module (CBM)" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 531, no. 2, 6 November 2002 (2002-11-06), pages 375-380, XP004391664 ISSN: 0014-5793

The present application relates to a method for the purification of recombinant fusion proteins comprising cellulose binding domain , where the purification method comprises reversible binding of the cellulose binding module under non-denaturing elution conditions and NO binding of the cellulose binding module , or its recombinant fusion protein to insoluble cell-wall plant material.

These fusion constructs for the purification of recombinant proteins produced in transgenic plants offer the unexpected advantage of selectively binding to the recombinant fusion

protein of interest , while avoiding contamination of binding plant derived cell-wall fragments or other insoluble plant solids .

A specific embodiment is the use of the cellulose binding domain CBM9-2 , with seq.1, from the hyperthermophile Thermotoga maritima . The process comprise the use of proteolysis with the bovine enterokinase with seq.2 , fused to this CBM9-2 sequence . During the proteolysis , the fusion enterokinase stably binds to the avicel affinity matrix, while the nonbinding recombinant protein , cleaved from its CBM9-2, is isolated . The CBM9-2 fusion enterokinase can later be released from the avicel and reused at a further stage. The CBM9-2 does appear not ti bind to plant derived cell-wall fragments or other insoluble solids from milled seed .

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Novelty(Article 33.2 PCT)

D1 relates to methods for separating and/or concentrating polypeptides , like recombinant proteins , by affinity phase separation using a polymer-ligand pair in which the ligand binds to a soluble phase-forming cellulose. The invention is exemplified by the use of a compound comprising as the affinity ligand a cellulose-binding domain from a Cellulomonas fimi cellulase .

A specific protease can be used for enzymatic removal of the compound from the polysaccharides binding moiety which remains bound to the oligosaccharide polymer by incorporating a protease recognition sequence between the compound and the polysaccharides binding moiety. Where cleavage is used, the protein of interest or chemical moiety can be cleaved readily from the polysaccharides binding region by the use of a protease specific for a sequence present between the polysaccharides binding region and the protein of interest leaving the polysaccharides binding peptide bound to the oligosaccharide polymer. Preferably, the protease is provided in a form which will facilitate its removal following cleavage of the polypeptide of interest from the polysaccharides binding peptide PBP. As an example, the cleavage protease can be prepared as a

cleavage enzyme complex, wherein the protease is itself bound to a polysaccharides binding moiety . This allows the proteolytic removal of the PBP from the fusion protein to liberate the protein compound of interest, while the fusion protease is still bound to the polysaccharides . This protease can be recycled by subsequent elution from the solid polysaccharides and reused .
Examples of biological which can be purified in this way include e.g Factor X or any recombinant polypeptide which can be fussed to a polysaccharides binding peptide (PBP) . Examples include culture broth (from prokaryotic or eukaryotic cell or tissue cultures), and extracts from cell lysates including plants , and hence implicitly lysates transgenic expressing recombinant polypeptides .
Generally, the mixture is clarified prior to application to the affinity partitioning system to remove cellular debris .
To define the affinity of the PBD-fusion proteins for different cellulose allomorphs, binding to various cellulosic matrixes can simply be evaluated by SDS-PAGE analysis of bound fractions. This analysis has shown that e.g. PBDN1 binds to amorphous cellulose (PASC) but does not bind to crystalline cellulose (BMCC). The CBDCenA on the other hand has affinity for both cellulosic materials..The fusion proteins can be Immobilized on Avicel cellulose . Affinity chromatography on cellulose was used to purify the polysaccharides binding peptide .
(see the abstract, column 1 paragraphs 2,3 ,summary of the invention , column 26 line 22 column 27 line 12 , column 28 lines 17 -28 , examples 6 and 8, and fig.7, 14)

However D1 does not disclose that the cellulose binding module is capable of binding reversibly to a polysaccharides matrix and being released from such a matrix by non-denaturing elution conditions and does not bind to insoluble cell-wall plant material . The recombinant fusion proteins do not bind to insoluble plant material .

Due to the fact that the subject-matter of claims 1-21 do comprise the following essential technical elements , being
- that the cellulose binding module is capable of binding reversibly to a polysaccharides matrix and is capable of being released from such a matrix by non-denaturing elution conditions
- that neither cellulose binding module , nor the recombinant fusion protein do not bind to insoluble cell-wall plant material ,

its subject matter is new in view of the prior art .

2. Inventive step(Article 33.3 PCT)

D2 discloses the provision of recombinant fusion protein (FP) recombinant protein (RP) and a cellulose binding peptide (CBP) being fused to it, expressed in transgenic plants . The transgenic plant are monocot or dicots .
a process of expressing a recombinant protein in a plant and isolating the recombinant protein from the plant comprises:
(a) providing a plant, a plant derived tissue or cultured plant cells expressing fusion protein including an heterologous protein and a cellulose binding peptide being fused to it, separated through a unique amino acid sequence recognizable and digestible by a protease
(b) homogenizing the plant, plant derived tissue or cultured plant cells such that FP is brought into contact with a plant derived cellulosic matter to effect affinity binding of the fusion protein via the cellulose binding peptide to cellulosic matter and forming a complex and
© isolating the FP-CM complex.
(d) proteolytic digestion of said fusion protein so as to release said recombinant protein
It discloses that xylanases can be a source of cellulose binding domains .
Where cleavage is used, the recombinant protein can be cleaved readily from the cellulosic matter by the use of a protease specific for a sequence present there between and the cellulose binding peptide.
Cellulose affinity purification is conducted as described in many prior art patent application .
Avicel 200 is used as binding reactant for the purification of protease- cellulose binding peptide fusion protein .
(see the abstract , page 34 line 28 page 37 line 34 , page 49 paragraph 4 , table 4 figures 1,2 and claims 1-24)

D3 discloses metal ion affinity peptide used in fusion protein for protein purification in immobilized Metal ion affinity chromatography .Fusion proteins comprising an enterokinase proteolytic site are disclosed . When the heterologous fusion protein is bound to the affinity column , the wild type protein is spliced with a bovine enterokinase fusion protein

comprising an affinity tag . The wild type protein is then selectively released from the column, while the fusion enterokinase still binds to it .
a variety of host-vector systems may be utilized to propagate and/or express the subject polynucleotides , including plant cell systems .
The bovine enterokinase shows 81.986% identity (81.986% ungapped) in 705 nt overlap (1-705:2305-3009) with seq.2 of the present application .
(see the abstract , figs 2,4 and page 6 left column paragraph 2)

D4 discloses expanded bed adsorption chromatography for the extraction of a desired native or recombinant protein from a plant in pure form . (see the abstract).

D1 is considered to be the closest prior art .

D1 differs from the subject-matter of claims 1-21 by the lack of
- reversible binding of the cellulose binding module under non-denaturing elution conditions
- binding to insoluble cell-wall plant material of the cellulose binding module , or its recombinant fusion protein .

The problem to be solved is the use of further purification methods using fusion polypeptide comprising a cellulose binding domain .

Due to the industrial importance of this problem ,the person skilled in the art would have had the incentive to solve this problem .

However knowing this problem of purification methods using fusion polypeptide comprising cellulose binding domain and knowing the prior art , the person skilled in the art would NOT have had reasonable expectation of success of using purification methods using a recombinant fusion protein comprising a cellulose binding module , where the purification method comprises
- reversible binding of the cellulose binding module under non-denaturing elution conditions
- NO binding to insoluble cell-wall plant material of the cellulose binding module , or its recombinant fusion protein .

The subject-matter of claims 1-21 is hence inventive .
These fusion constructs for the purification of recombinant proteins produced in transgenic plants offer the unexpected advantage of selectively binding to the recombinant fusion protein of interest , while avoiding contamination of binding plant derived cell-wall fragments or other insoluble plant solids .

Re Item VIII

Certain observations on the international application

D1 discloses that different cellulose binding module fusion proteins have different affinities to different cellulose allomorphs,

D8 discloses the biochemical characterization of Thermotoga maritima endoglucanase Ce174 that does not comprise a carbohydrate binding module (CBM) .
However Tm Cel74 was binding to active towards barley glucan and to a lesser extent to carboxymethyl cellulose (CMC) . Tm Cel74 did not contain a carbohydrate binding module (CBM), versions of which have been identified in the amino acid sequences of other family 74 enzymes. (see the abstract)

The term 'substantially' used in claim1 is vague and unclear and leaves the reader in doubt as to its exact technical meaning . In view of D1 and D8 , and in view of above mentioned unclarity , the functional generic term 'cellulose binding module CBM ... not binding *substantially* to insoluble cell-wall plant material ' used in claim 1 is vague and unclear .

CLAIMS

1. A process for purification of a heterologous protein of interest, comprising

(a) providing a fusion protein comprising said heterologous protein fused to a CBM intercepted by a proteolytic cleavage site,
(b) contacting said fusion protein with a functional protease fused to a CBM , at conditions facilitating proteolytic cleavage by said protease, to cleave the CBM from the heterologous protein of interest,
(c) contacting the solution of CBM-protease, free CBM and heterologous protein of interest to a polysaccharide matrix, under conditions where the CBM-protease and free CBM binds to said polysaccharide matrix and where the heterologous protein of interest is not retained on said polysaccharide matrix,
(d) separating the non-bound heterologous protein of interest from the polysaccharide matrix,
(e) washing the polysaccharide matrix with the bound CBM-protease and CBM, with one or more suitable aqueous solutions,
(f) eluting the CBM-protease from the matrix by adjusting conditions effecting the release of said CBM-protease off the matrix; and
(g) optionally reconditioning said eluted CBM-protease, to retain its affinity to said polysaccharide matrix, such that the reconditioned CBM-protease can be re-used for subsequent repetition of the process defined by steps (a) – (g)
wherein said CBMs are capable of binding reversibly to a polysaccharide matrix and being released from such matrix by non-denaturing elution conditions and do not bind substantially to insoluble cell-wall plant material, the method.

2. The process of claim 1, wherein said protease fused to CBM is from the group of proteases cconsisting of enterokinase, tobacco etch virus (TEV) protease, factor X and thrombin.

3. The process of claim 2 wherein said protease is mammalian enterokinase (EK) or an enterokinase active part thereof.

4. The process of claim 3, wherein said EK comprises a bovine EK catalytic domain (EKc).

5. The process of claim 4, wherein said bovine EKc is encoded by the nucleic acid sequence shown as SEQ ID NO: 2.